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Phospholipid Composition and Organization in Model β-Thalassemic Erythrocytes

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The membrane phospholipid organization in human red blood cells (RBC) is rigidly maintained by a complex system of enzymes. However, several elements of this system are sensitive to oxidative damage. An important component in the destruction of β-thalassemic RBC is the generation of reactive oxygen species and the release of redox-active iron by the unpaired α-hemoglobin chains. Consequently, we hypothesized that the presence of this oxidative stress to the RBC membrane could lead to alterations in membrane lipid organization. Model β thalassemic RBC, prepared by the introduction of excess α-globin in the cell, have previously been shown to exhibit structural and functional changes almost identical to those observed in β-thalassemic cells. After 24 hr at 37°C, the model β thalassemic cells exhibited a significant loss of deformability, as measured by ektacytometric analysis, indicative of extensive membrane damage. However, a normal steady-state distribution of endogenous phospholipids was found, as evidenced by the accessibility of membrane phospholipids to hydrolysis by phospholipases. Similarly, the kinetics of transbilayer movement of spin-labeled phosphatidylserine (PS) and phosphatidylethanolamine (PE) in all samples was in the normal range and was not affected by the presence of excess α-globin chains. In contrast, a faster rate of spin-labeled phosphatidylcholine (PC) transbilayer movement was observed in these cells. While control RBC exhibited a complete loss of their initial (2 mol%) lysophosphatidylcholine (LPC) levels following 24 hr of incubation at 37°C, 1.5 mol% LPC was still present in model β-thalassemic cells, suggesting an altered phospholipid molecular species turnover, possibly as a result of an increased repair of oxidatively damaged phospholipids.

Key words: thalassemia, red cells, phospholipids, membrane

INTRODUCTION

The phospholipid molecular species composition of the red blood cell (RBC) is complex [1] and, although well maintained during the lifetime of the cell, involves a dynamic deacylation/reacylation process that results in a continuous turnover of the fatty acyl groups. In addition, the phospholipids of the RBC membrane are distributed asymmetrically between the inner and outer leaflets of the lipid bilayer. Whereas the choline-containing phospholipids, phosphatidylcholine (PC) and sphingomyelin (SM), are localized mainly in the outer monolayer, the aminophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS), are found mainly or exclusively in the inner monolayer [2].

This asymmetric architecture of the membrane phospholipids is dynamic and reflects the differential equilibrium of the movement of phospholipid molecular species across the bilayer. The rate of transbilayer movement is dependent on the phospholipid class as well as molecular species within each class. The aminophospholipids are actively transported by an adenosine triphosphate (ATP)-dependent translocation system, the aminophospholipid translocase, or "flipase" [3–6], which has a high affinity for PS. In addition, all phospholipid classes are also transported independent of ATP, at rates significantly slower than the ATP-dependent translocase, strongly affected by the acyl chains of the phospholipid molecular species [7,8]. Consequently, damage to lipid subclasses or any of the mechanisms involved in maintaining the lipid

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asymmetry or packing of the RBC may lead to perturbations in the structure and function of the RBC membrane.

Oxidant-mediated injury is one mechanism by which the structure and function of the lipid bilayer may be altered. Much of this damage results from changes to the phospholipid molecular species composition as a consequence of direct oxidative attack on the polyunsaturated acyl chains. The recognition, removal, and repair of these damaged lipids is another primary mechanism involved in the maintenance of the phospholipid composition and asymmetry of the RBC. Indeed, we recently showed that oxidant-mediated changes in lipid packing can be recognized by phospholipases [9]. Hence, cellular oxidation may significantly alter the structure of the lipid bilayer via direct oxidative injury to the lipid components or via the inactivation of the normal homeostatic mechanisms involved in maintaining the bilayer asymmetry.

Oxidant injury has also been implicated as an important component in the pathophysiology of the β-thalassemic RBC [10]. This hypothesis is supported by the observation that isolated α-hemoglobin chains directly generate reactive oxygen species [11,12], lead to elevated levels of globin free heme and iron in the membrane, and decrease membrane sulfhydryl content [13–15]. Increased interaction of globin chains with the cytoskeleton has been reported [10,16,17], and increased prothrombinase activity indicated the exposure of PS in the red cell bilayer [18]. To investigate whether the increased oxidant stress present in the thalassemic RBC might alter the membrane phospholipid organization and/or repair of damaged phospholipids, normal RBC were loaded with purified α-hemoglobin chains [19]. These model β thalassemic cells have previously been shown to exhibit changes in RBC structure and function virtually identical to those from individuals with β-thalassemia [20–22] and indicate the occurrence of oxidative membrane damage [23]. The effect of entrapped α-hemoglobin chains on the organization of endogenous phospholipids, the redistribution kinetics of spin-labeled phospholipids introduced into the outer monolayer, and the incorporation of free fatty acid into phospholipids were examined.

MATERIALS AND METHODS

Erythrocytes

After informed consent was obtained, human erythrocyte suspensions were prepared from fresh venous blood collected in heparinized tubes. Blood was filtered through cellulose as described by Beutler [24] to remove white cells and platelets. Erythrocytes were pelleted by centrifugation and washed thrice with 0.9% NaCl. The erythrocytes were loaded with purified α-chains by osmotic lysis and resealing as described previously [19]. Three populations of cells were prepared: 1) control RBC that did not undergo lysis and resealing; 2) control-resealed RBC that were lysed and resealed in the absence of α-hemoglobin chains; and 3) model β thalassemic RBC in which purified heme containing α-hemoglobin chains (referred to hereafter as α-hemoglobin chains) were entrapped (10 mg α-hemoglobin chains/ml pRBC). The final intraerythrocytic α-chain concentration was 3.8% ± 0.5% of total hemoglobin. The heme-containing α-subunit of normal hemoglobin A (α2β2) was prepared as previously described [20,25,26]. The cells were washed and subsequently incubated in Hank's balanced salt solution (HBSS; Sigma, St. Louis, MO) at 37°C at a hematocrit of 10%. At the specified time points, sample aliquots were removed, washed, and resuspended in appropriate buffers for the analyses described below. The buffer used for the phospholipase experiments contained 50 mM NaH_2PO_4, 4.95 mM KCl, 1.25 mM KH_2PO_4, 0.25 mM $MgCl_2$, 0.5 mM $CaCl_2$, 120 mM NaCl, pH 7.4. The buffer for the spin labeling and incubation studies was composed of 10 mM HEPES, pH 7.4, containing 144 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 10 mM glucose, 5 mM KCl. The incorporation of arachidonic acid was done in HBSS.

Measurement of Cellular Deformability

The ektacytometric osmotic deformability profile of intact erythrocytes was determined by using a Technicon-ektacytometer (Technicon Instruments, Tarrytown, NY) which continuously monitors the laser diffraction pattern of RBC deformed in a viscometer at a constant shear stress of 120 dynes/cm^2 as a function of the osmolality of the medium (50–500 mosmol/kg) [27]. The instrument has been described in detail previously [28,29] and was interfaced to a Macintosh computer (Apple Computer, Inc., Cupertino, CA) for automated data analysis.

Lipid Analysis

Erythrocyte membrane lipids were extracted according to the method of Rose and Oklander [30]. Phospholipid classes were separated by two-dimensional thin-layer chromatography (TLC) according to the method of Roelofsen and Zwaal [31], and lipid phosphorus was determined according to the method of Rouser et al. [32].

Determination of the Transmembrane Distribution of Endogenous Phospholipids

The hydrolysis of membrane phospholipids by phospholipase A_2 from bee venom (Sigma) and sphingomyelinase C from *Staphylococcus aureus* (Sigma) was determined as described by Roelofsen and Zwaal [31]. Subsequent to the incubation with the phospholipases and before the addition of EDTA, to stop the phospholipase action, erythrocytes were pelleted by centrifugation, and the relative amount of hemoglobin in the supernatant was used to calculate the percentage of cells that hemolysed during phospholipase treatment. Based on the accepted assumption that all phospholipids in a cell become avail-

able for phospholipase hydrolysis as hemolysis occurs, a mathematical correction was made to determine the fraction of phospholipid in the outer monolayer: $PL_{out} = 100 \cdot (A - H)/(100 - H)$, in which PL_{out} = percentage of phospholipid on the outside, A = percentage of phospholipid hydrolyzed, and H = percentage of hemolysis. This correction was less than 2%, as a result of the low level of hemolysis in all samples tested (2–3%).

Determination of the Transmembrane Redistribution of Spin-Labeled Lipids

The transmembrane movement of membrane phospholipid classes (PC, PE, and PS) was determined using spin-labeled phospholipids and their subsequent extraction from the outer monolayer of labeled RBC by bovine serum albumin (BSA) [33]. The spin-labeled phospholipids used contain a short nitroxide-labeled fatty acyl group in the sn-2 position. Before being labeled, erythrocytes (30% hematocrit) were incubated for 5 min in buffer at 37°C with 5 mM (final concentration) diisopropyl fluorophosphate (DFP) to minimize the hydrolysis of the spin-labeled phospholipids [33]. The spin-labeled phospholipids used were: 1-palmitoyl-2-(4-doxyl-pentanoyl)-phosphatidylcholine (spPC); 1-palmitoyl-2-(4-doxyl-pentanoyl)-phosphatidylethanolamine (spPE); and 1-palmitoyl-2-(4-doxyl-pentanoyl)-phosphatidylserine (spPS). These probes were synthesized as described by Morrot et al. [34]. The spin-labeled phospholipids were dried from a chloroform solution and resuspended in buffer [33]. Labeling was carried out at a 30% hematocrit with the label at a final concentration of approximately 10 μM. Determination of the amount of spin label in the outer monolayer of the erythrocyte was performed using a back-exchange technique with BSA as described elsewhere [33]. Erythrocytes were separated from the spin-labeled BSA supernatant by a 1 minute spin at 7,600*g* in an Eppendorf centrifuge. Quantification of the spin-label of the supernatants was performed on a Jeol RE1X EPR spectrometer.

Determination of Arachidonic Acid Turnover

Radiolabeled arachidonic acid (specific activity 208 μCi/nmole), dissolved in 2 μl ethanol, was added to 450 μl HBSS to obtain a final concentration of 1 nM arachidonic acid. An equal volume (i.e., 450 μl) of a 20% washed RBC suspension in HBSS was added, and the mixture was slowly stirred at 37°C. Samples were taken at appropriate time intervals, and RBC were pelleted. Aliquots of the total mixture, supernatant, and pellet were taken to determine the distribution of radioactivity between RBC and surrounding medium. The RBC were washed in 0.9% sodium chloride, and lipids were extracted according to Rose and Oklander [30]. The radioactivities in the different phospholipid fractions and free fatty acid were determined after separation by TLC (Silicagel HL; Analtech, Newark, DE) in $CHCl_3/MeOH/H_2O/AcOH$ (100:44.4:2.2:13.3, v/v).

ATP Measurement

Intracellular ATP concentration was measured in a 30% cell suspension in the buffer used for the spin-label studies. ATP was extracted from the cells in 0.5% trichloroacetic acid—2 mM EDTA and measured with the Pharmacia LKB luciferin-luciferase assay [35].

RESULTS

Characterization of Erythrocyte Deformability

It has previously been shown that entrapment of α-chain in normal RBC has a profound effect on intracellular oxidant stress, which in turn is correlated with significant changes in the membrane characteristics of the red cell, including oxidative damage to proteins and lipids [19–23]. Because our previous studies have demonstrated that oxidative injury will strongly affect the deformability erythrocytes [36], we evaluated the ektacytometric osmotic deformability pattern of the control, control-resealed, and model β-thalassemic erythrocytes. Typical osmotic deformability profiles are given in Figure 1A,B. The curves exhibit a characteristic maximum deformability (DI_{max}) at isotonicity (290 mosmol). Because the DI_{max} of erythrocytes from different individuals varies, we normalized this value, as measured in different experiments, relative to that of fresh, untreated RBC (Fig. 1C). Whereas the process of osmotic lysis and resealing decreased the deformability of the RBC (Fig. 1A,C), the DI_{max} of the control and control resealed cells declined only slightly after incubation for 24 hr in buffer at 37°C (Fig. 1B,C). In contrast, the deformability pattern and DI_{max} of the α-chain-loaded cells changed significantly during the same incubation period ($P = 0.005$, t test; Fig. 1B,C). Similar shifts in the deformability pattern were observed in thalassemic cells [17] and red cells exposed to oxidative stress [36]. Hence, the change in the deformability profile of the model β-thalassemic cells clearly indicates significant membrane alterations that could be the result of cumulative membrane oxidative damage [23].

Composition and Transbilayer Distribution of Endogenous Phospholipids

Previous studies [10,13–17,21,22] have demonstrated significant alterations in cytoskeletal components of the β- and model β-thalassemic RBC, and it was recently reported that the hypercoaguable state in thalassemic patients might be due to PS exposure in the red blood cell membrane [18]. Few data exist, however, regarding whether significant changes in the lipid bilayer (composition and asymmetry) result from the unpaired α-hemoglobin chains. Consequently, we have determined the relative amounts of the different phospholipid classes as well as

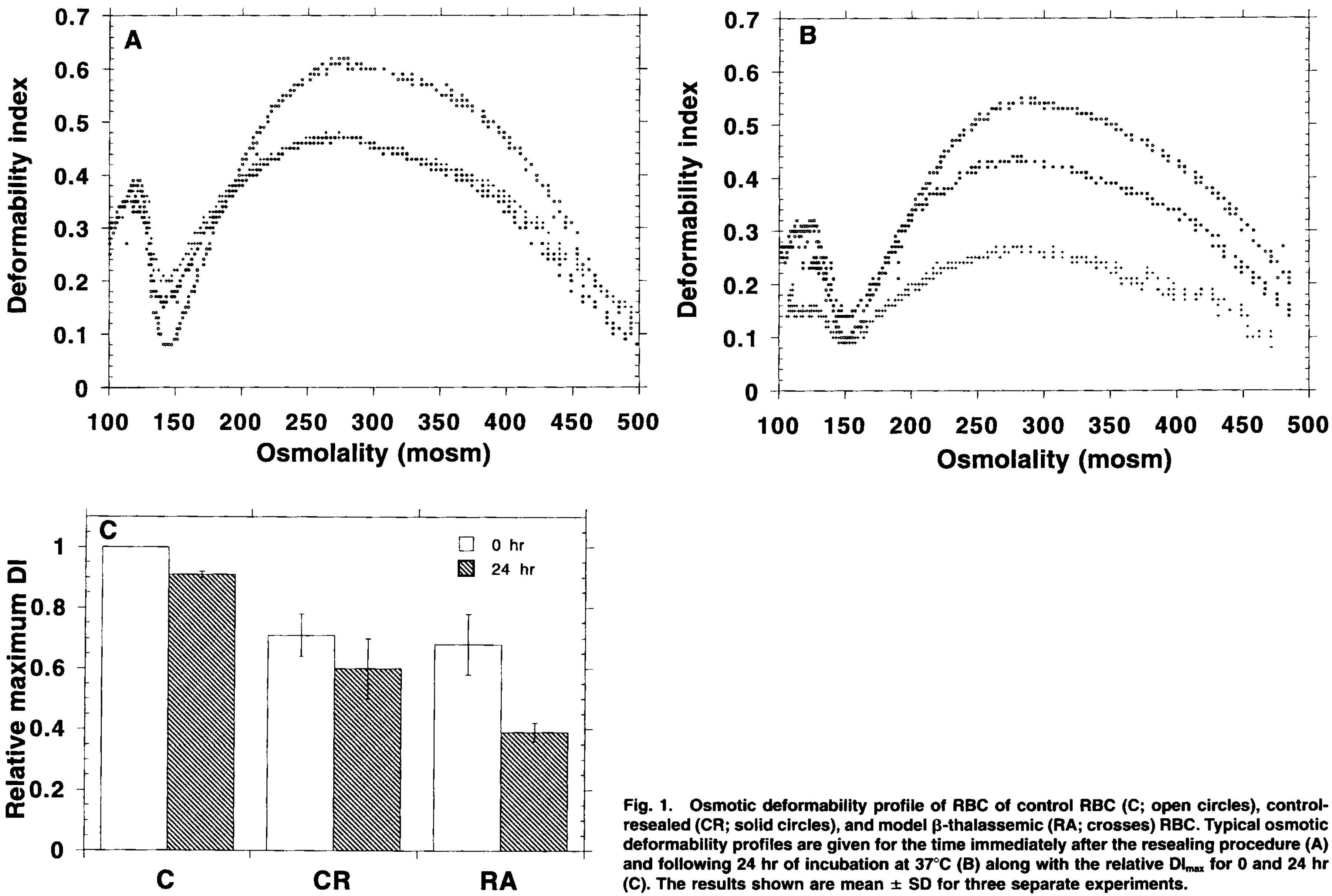

Fig. 1. Osmotic deformability profile of RBC of control RBC (C; open circles), control-resealed (CR; solid circles), and model β-thalassemic (RA; crosses) RBC. Typical osmotic deformability profiles are given for the time immediately after the resealing procedure (A) and following 24 hr of incubation at 37°C (B) along with the relative DI_{max} for 0 and 24 hr (C). The results shown are mean ± SD for three separate experiments.

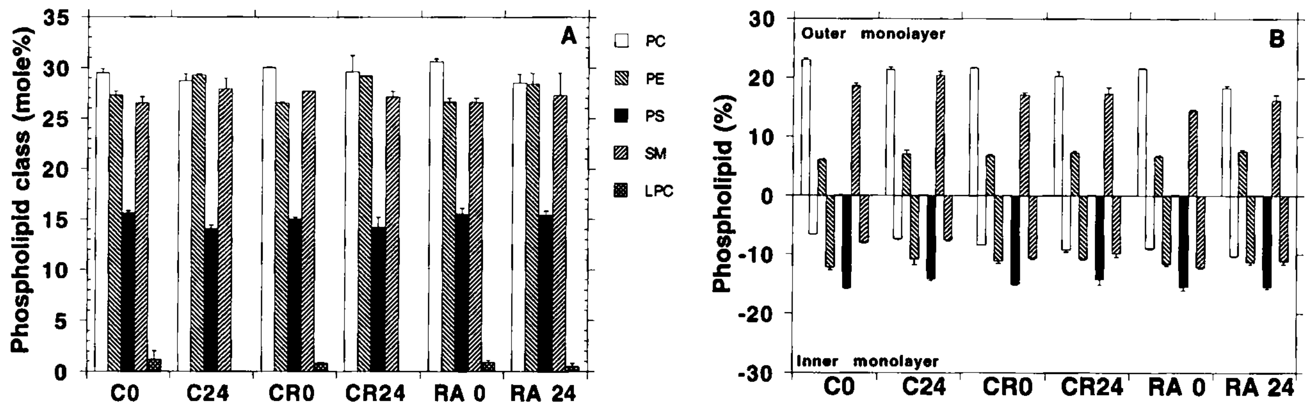

Fig. 2. Relative amounts of the major phospholipids and the distribution of endogenous PC (□), PE (▧), PS (■) and SM (▨) between the inner and outer monolayers. Shown are control RBC (C), control-resealed (CR), and model β-thalassemic (RA) RBC immediately after the resealing procedure (C0, CR0, RA0) and after 24 hours of incubation at 37°C (C24, CR24, RA24). The averages and SD of eight samples are shown.

their distribution between inner and outer monolayers of the red cell membrane (as determined by availability to phospholipase degradation). As is shown in Figure 2, no significant differences were found in the phospholipid composition of control, control-resealed, or α-chain-loaded RBC at time zero or after incubation for 24 hr at 37°C. However, although in control and control-resealed RBC the lysophosphatidylcholine (LPC) present at time zero had disappeared after 24 hr incubation at 37°C, LPC was still present in the α-chain-loaded cells (Fig. 2A). This observation suggested a disorder in the acylation of LPC to PC in the model thalassemic RBC membrane.

The asymmetric distribution of phospholipid in the bilayer of the control and model β-thalassemic RBC was also examined using a combination of phospholipase A_2 from bee venom and sphingomyelinase C from *S. aureus* (Fig. 2B). All cells exhibited less than 3% hemolysis after treatment with both enzymes. The results of these experiments showed only small differences in the accessibility of PC, PE, and SM to phospholipase hydrolysis, and no PS degradation was observed in any of the samples. These results, similar to results from previous reports on normal erythrocytes [37], indicate that α-hemoglobin chain loading has no effect on the asymmetric organization of endogenous phospholipids in the erythrocyte.

Transbilayer Kinetics of Spin-Labeled Phospholipid Analogs

Because much of the flip-flop of phospholipids across the membrane is an energy-dependent, protein-mediated event, cellular/membrane oxidation might be expected to alter this mechanism of maintaining bilayer composition and asymmetry. Indeed, previous studies have indicated that the aminophospholipid translocase is oxidant sensitive [38,39]. Therefore, we examined the transbilayer movement of phospholipids using spin-labeled phospholipid analogs. A typical result of the redistribution of spin-labeled phospholipids across the membrane bilayer of normal cells is shown in Figure 3A. The lines connecting the experimental data points are derived from a least-square, exponential nonlinear regression fit [$y = a + b \cdot \exp(-k \cdot t)$]. The half-times of equilibration across the bilayer, as calculated from these fits, are approximately 4.5, 25, and 150 min for spPS, spPE, and spPC, respectively. The plateaus, as calculated from these fits, that were reached for the distribution across the bilayer for PE (79%) and PC (20%) closely resembled the normal asymmetric distribution for these phospholipids based on their availability to phospholipase degradation as depicted in Figure 2. The redistribution kinetics of spin-labeled PS indicates that approximately 10% of the spPS introduced into the membrane resides in the outer monolayer at equilibrium and is extractable by BSA. In contrast, no endogenous PS could be hydrolyzed by phospholipases (Fig. 2). These data on rate and equilibrium distribution of spin-labeled phospholipids are in agreement with published results [33], and the difference between the equilibrium distribution of spin-labeled PS and the availability of endogenous PS to phospholipases was noted previously [5]. The data on the kinetics of spin-labeled phospholipid redistribution of erythrocytes in association with osmotic lysis and resealing in the absence or presence of α-hemoglobin chains are given in Figure 3B–D. For spPS and spPE (Fig. 3B,C), the differences between control-resealed cells and those loaded with α-chains were very minor after incubation for 24 hr at 37°C. In contrast, spPC in cells loaded with α-chains seemed to show an increased movement to the inner monolayer compared to resealed cells or control RBC (Fig. 3A,D).

The activity of the aminophospholipid translocase can

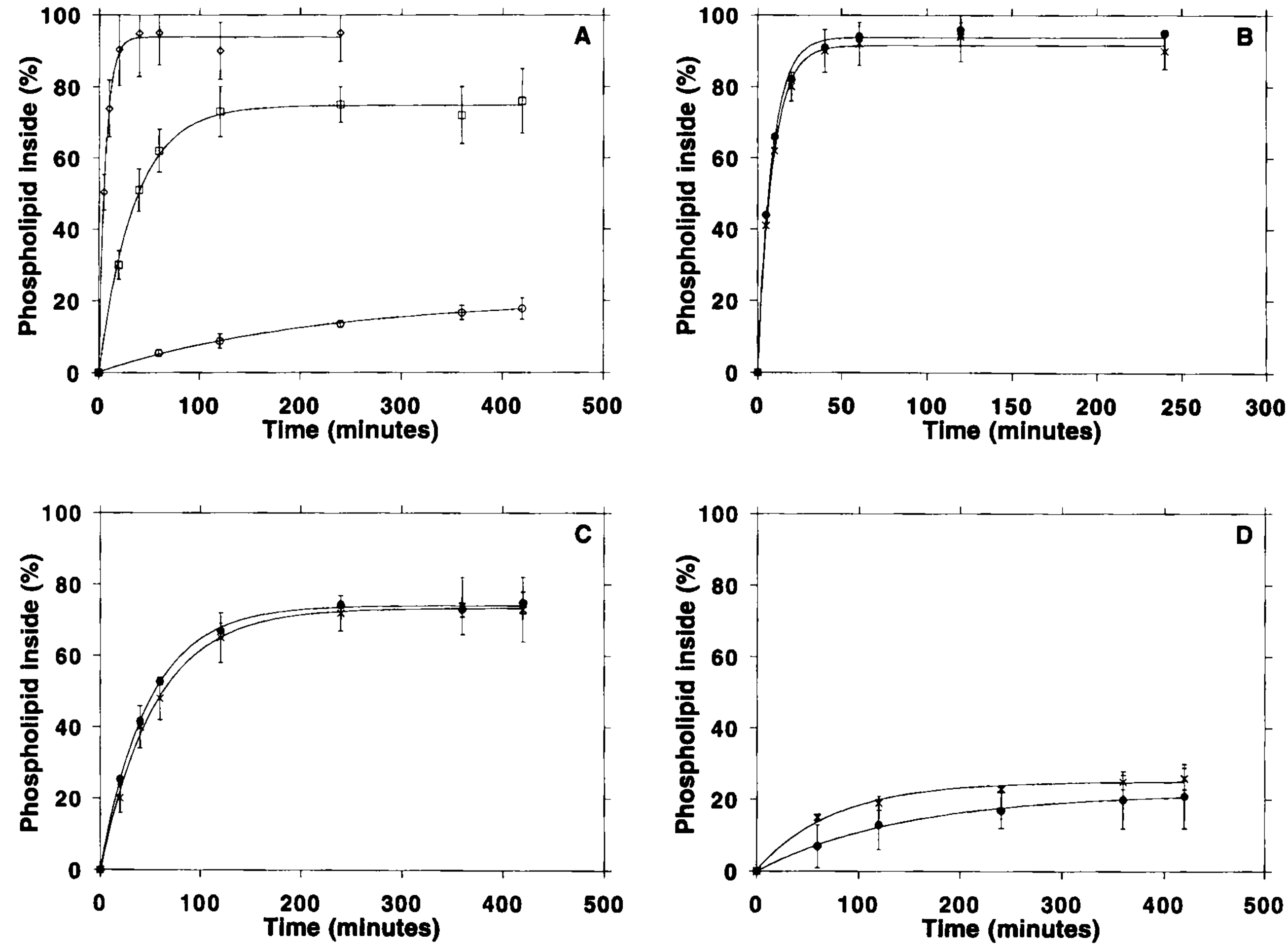

Fig. 3. A: Typical kinetics of the inward translocation of spin-labeled PS (diamonds), PE (squares), and PC (open circles) in membranes of control RBC that have not undergone the lysis resealing procedure after incubation for 24 hr at 37°C. B–D: Typical kinetics of the inward translocation of spin-labeled PS (B), PE (C), and PC (D) in membranes of RBC that have undergone the lysis resealing procedure in the absence of α-chains (solid circles) and RBC that were loaded with α chains (crosses) after 24 hr of incubation at 37°C. The curves of three samples shown were fitted to the experimental data by least square, exponential nonlinear regression [$y = a + b \cdot \exp(-k \cdot t)$].

also be affected by ATP depletion [5]. We therefore tested the intracellular ATP concentration of the samples used for the redistribution studies with the spin-labeled probes. The ATP level was similar in all samples (average value 0.99 ± 0.15 mM) and did not differ significantly from the ATP levels determined in the normal control cells (1.03 ± 0.08 mM). No correlation was found between the ATP levels in the cells tested and the kinetics of transbilayer movement, probably because the variation is not substantial enough to affect the aminophospholipid translocase. In summary, the active movement of the spin-labeled aminophospholipids PS and PE was not significantly affected by the presence of α-hemoglobin chains after 24 hr of incubation at 37°C, indicating normal activity of the aminophospholipid translocase. However, the transbilayer movement of PC seemed somewhat increased in the model β-thalassemic cells, indicating organizational changes in the membrane affecting the non-ATP-dependent movement of phospholipids. The difference is however small and should be treated carefully given the limited number of samples (n = 3).

Phospholipid Turnover

The difference in the LPC level in the three different cell types at 24 hr of incubation at 37°C (Fig. 2A) suggested a disorder in the normal phospholipid turnover in the RBC membrane. In the absence of an exogenous LPC pool (plasma), no new exogenous lysophospholipid is introduced into the membrane. The lysophospholipid pool present in the membrane of normal cells is depleted by acylation to phospholipid. Hence, the presence of LPC in α-chain loaded cells could be the result of an impaired acylation process of LPC to PC or an enhanced breakdown of PC by phospholipase to LPC under these conditions.

When a trace amount of radiolabeled arachidonic acid

was added to RBC immediately after the lysis and resealing process, it was rapidly incorporated into the membrane as fatty acid (FA) and was subsequently found in the phospholipid fraction (Fig. 4A). After a rapid first phase, this formation of radiolabeled phospholipids progressed slowly during 30 hr of incubation at 37°C. The relative distribution after 30 hr of incubation in the different lipid fractions is given in Figure 4C. Arachidonic acid is incorporated into all major glycerophospholipids (PC, PE, PS), with PC being the metabolically most active. Virtually no radioactivity was found in the SM fraction. Only minor differences can be observed between control, control-resealed, and model β-thalassemic RBC, and these indicate an active reacylation system, including the formation of acylCoA by the long-chain acylCoA synthase and the transacylation into PL by the acylCoA-lysophospholipid acyltransferase. These data demonstrate that unpaired α-hemoglobin chains do not inhibit the incorporation of arachidonic acid into phospholipid.

However, after 30 hr, major differences were observed between control, control resealed, and α-chain-loaded cells. When a trace amount of radiolabeled arachidonic acid was added to RBC after 30 hr of incubation at 37°C, the incorporation process was significantly impaired in control and control-resealed cells (Fig. 4B), whereas the α-hemoglobin chain-loaded cells rapidly incorporate arachidonic acid in the phospholipid fraction (Fig. 4B), indicating that these cells still contained an active reacylation system. When cells were stored at 4°C for 30 hr, LPC was not depleted and arachidonic acid was rapidly incorporated at 37°C in all cells (not shown). In addition, ghost membranes prepared from control cells incubated overnight at 37°C that lacked LPC and ghosts from fresh RBC showed virtually identical acylCoA synthase activity and acylCoA-LPC acyl transferase activity (not shown), indicating that the enzymes of the reacylation process are not impaired in the control cells. These data suggest that the lack of lysophospholipid, the substrate for acylCoA-lysophospholipid acyltransferase, is the underlying reason for the impaired incorporation of arachidonic acid in control and control-resealed cells. These data again suggest that the model β-thalassemic cells in contrast to control cells, were generating significant amounts of LPC, perhaps as a consequence of the ongoing oxidant stress arising from the unpaired α-hemoglobin chains.

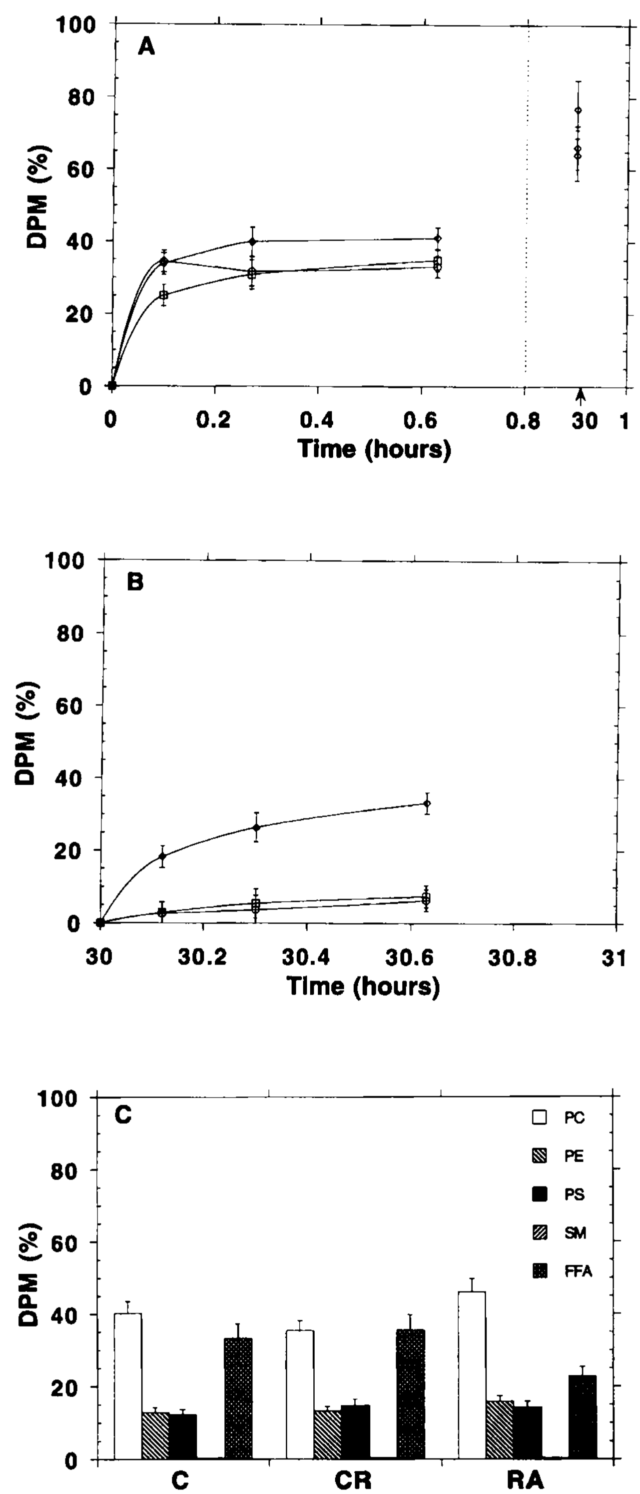

Fig. 4. Radioactivity in the phospholipid fraction of the RBC membrane lipids during the incubation with radioactively labeled arachidonic acid of control RBC (circles), control-resealed (squares), and model β-thalassemic (diamonds) RBC. A: Incubation with arachidonic acid added immediately following the resealing procedure. B: Incubation with arachidonic acid added after 30 hr of incubation at 37°C. C: The relative distribution of radioactivity labelled arachidonic acid in membrane lipids after 30 hr of incubation.

DISCUSSION

The phospholipid composition and organization of human RBC are very complex and include more than 200 glycerophospholipid molecular species [1], which are asymmetrically distributed in the bilayer. This phospholipid organization is very dynamic and includes active processes that maintain the asymmetric organization [6]

as well as deacylation and reacylation processes that result in a continuous turnover of the fatty acyl groups [39]. The composition and distribution of these lipids are well maintained throughout the 120 day life of the cell despite the relatively high level of oxidant stress to which the unsaturated fatty acyl groups are subjected in the normal RBC.

However, under certain conditions (e.g., sickle cell anemia and the thalassemias), the oxidant defense system of the RBC can be overwhelmed and/or circumvented, leading to oxidant-mediated lipid and protein damage. Oxidatively modified phospholipids will alter normal membrane structure and function, and their recognition and removal, as well as the subsequent restoration of damaged phospholipid molecular species, are therefore of prime importance to the RBC. Whereas these repair processes maintain phospholipid organization under oxidative stress, the enzymes involved are also themselves sensitive to oxidative damage. The key enzyme in maintenance of phospholipid asymmetry, the aminophospholipid translocase, contains an N-ethylmaleimide (NEM) sensitive sulfhydryl group essential for activity [38,39]. In addition, the rate-limiting enzyme in the reacylation process, acylCoA-lysophospholipid acyltransferase, is also inhibited by NEM [39]. Hence, in situations of high oxidative stress, those enzymes responsible for repairing oxidative damage can also be oxidatively inhibited, resulting in an altered phospholipid bilayer, with detrimental effects for the function of the cell.

Because both the distribution and composition of the lipid bilayer are sensitive to oxidation, it is possible that RBC characterized by a chronic oxidant stress might exhibit changes in the phospholipid bilayer. β-Thalassemic RBC are characterized by both a high rate of oxidant generation and the release of redox-active heme and iron into the membrane. We hypothesized that the presence of these powerful oxidative agents and catalysts in the membrane of β-thalassemic cells could lead to alterations in membrane phospholipid composition and organization. To investigate this hypothesis, we studied normal erythrocytes in which purified heme-containing α-hemoglobin chains were entrapped. These model β thalassemic RBC exhibit structural and functional changes almost identical to those seen in RBC obtained from individuals with β-thalassemia [13–15,19–23].

Our studies show that the model β-thalassemic cells undergo a significant alteration in their ektacytometric deformability pattern, indicative of membrane damage (Fig. 1). Although the osmotic deformability pattern (primarily the DI_{max}) of the control-resealed RBC was slightly altered following the resealing procedure, no further change was observed in these cells following prolonged incubation at 37°C. In contrast, the α-chain-loaded cells showed a significant time-dependent decrease in cellular deformability, which was correlated with increased oxidation of membrane and cytosolic components [19–23]. Our previous studies have demonstrated that similar changes in the deformability of normal cells can be induced by oxidants such as hydrogen peroxide and cumene hydroperoxide [36], and similar changes were reported for thalassemic cells [17]. Because both the distribution and the composition of the lipid bilayer are sensitive to oxidation, and this in turn can have a significant effect on membrane deformability, the structure and function of the lipid bilayer in model β-thalassemic cells were investigated.

The relative amounts of the major phospholipid classes did not significantly change in the model β-thalassemic cells (Fig. 2A). In addition, the accessibility of membrane phospholipids to hydrolysis by phospholipases was within the normal range (Fig. 2B) in the α-hemoglobin-loaded RBC. Furthermore, the kinetics of transbilayer movement of the spin-labeled PS and PE in all samples were in the normal range and were not affected by the presence of excess α-globin chains. These data indicate a normal steady-state distribution of endogenous phospholipids and normal activity of the aminophospholipid translocase (Fig. 3). In contrast, the redistribution of PC in model β-thalassemic cells after 24 hr of incubation at 37°C was increased, suggesting some membrane damage affecting the passive movement of phospholipids across the bilayer. A similar redistribution of PC has been observed in membranes oxidatively damaged by diamide [40] and in the mouse β-thalassemic RBC [41]. Importantly, as observed in this study, the aminophospholipid translocase appeared to have a normal activity, suggesting that the modifications in the transverse lipid organization were due to changes in the passive phospholipid diffusion. A reported stimulation of prothombinase activity by thalassemic cells indicated the presence of PS in the outer monolayer [18], and it was suggested that an impaired aminophospholipid translocase was responsible for the observed alteration [18]. Previous studies on stored red cells indicated that even a severely impaired aminophospholipid translocase activity does not necessarily lead to a loss of phospholipid asymmetry [42]. In addition to an impaired flipase activity, an increased scrambling of the bilayer seems to be needed, as is found in sickle cells [43] or as the result of calcium loading of red cells [44]. Our studies do not indicate a major loss of aminophospholipid translocase activity due to the increase in α-hemoglobin or an overall loss of phospholipid asymmetry or PS exposure as measured by the availability of phospholipids to phospholipases in the α-chain-loaded red cells.

In addition to an increased passive transbilayer movement of PC, phospholipid fatty acyl turnover also appears to be affected in the α-hemoglobin chain-loaded RBC. While control and control-resealed RBC exhibited a complete loss of their initial LPC levels (2 mol%) following 24 hr of incubation at 37°C (Fig. 2A), 1.5 mol% LPC was still present in model β-thalassemic cells, indicating

an altered turnover of LPC to PC or an increased formation of LPC. To examine this, radiolabeled arachidonic acid was added to the control, control-resealed, and model β-thalassemic RBC. Free fatty acid was rapidly incorporated in the phospholipids, including PC, of all samples (Fig. 4). The distribution of arachidonic acid within the different phospholipid classes was similar in the different samples after 30 hr of incubation (Fig. 4C). The incorporation process involves the formation of acylCoA from the free fatty acid by a long-chain acylCoA synthase and the transfer of the acyl group from acylCoA to the lysophospholipid by acylCoA-lysophospholipid acyltransferase. Our results clearly indicate that this reacylation process was not inhibited by the unpaired α-hemoglobin chains. On the contrary, the model β-thalassemic RBC incorporated even more arachidonic acid during the first 30 hr of incubation at 37°C (Fig. 4). In addition, while the control and control-resealed RBC exhibited a significant loss of their ability to incorporate free fatty acid after incubation for 30 hr at 37°C, active reacylation was still present in model β-thalassemic cells (Fig. 4B). This inability of the control and control-resealed RBC to incorporate free fatty acid after 30 hours was directly coupled to the availability of LPC (i.e., there was no LPC); the activities of the acylCoA synthase and acylCoA-lysophospholipid acyltransferase were not changed in membranes of control cells. Exogenously added fatty acid was rapidly incorporated into phospholipid of α-hemoglobin chain-loaded cells. Hence, exogenously added fatty acid can reach the relevant ligase and acyltransferase in these cells. We cannot completely rule out the possibility that endogenous fatty acid would be excluded from the metabolically active pool during the loading with α-chains and consequently limit the acylation of LPC. However, this exclusion would be specific for α-chain loading (because controlled resealed cells do not show this) and does not occur during the incubation after the loading of the cells, because radioactive fatty acid would be affected in the same way, which does not seem to be the case. Thus, an increased formation rather than a limited acylation seems the underlying reason for the increased levels of LPC observed in α-chain-loaded cells. In previous studies we have shown that oxidized phospholipids are preferentially hydrolyzed by phospholipases [9]. Hence, we suggest that in α-chain-loaded cells the higher level of lipid peroxidation [23] will lead to the increased formation of lysophospholipid.

In conclusion, although excess heme-containing α-globin chains led to membrane damage, as evidenced by altered cellular deformability, neither the endogenous phospholipid distribution nor the activity of the aminophospholipid translocase was significantly altered. Oxidative damage to both proteins and lipids has been observed in these cells [23]. Our results indicate that membrane protein damage, rather than disorganization of the lipid bilayer, accounts for the loss of cellular and membrane deformability seen in β- and model β-thalassemic RBC [22]. Indicative of elevated endogenous oxidation, our data demonstrate an increased turnover of the phospholipid molecular species in the model β-thalassemic RBC but a normal reacylation process. This suggests an increased formation of LPC as the result of the unpaired α-hemoglobin chains. The increased LPC generation may be the direct result of oxidative attack on the unsaturated phospholipids.

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